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Synergistic Lysis of Erythrocytes by *Propionibacterium acnes*

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Sheep and human erythrocytes, partially processed by *Staphylococcus aureus* or *Clostridium perfringens*, were susceptible to lysis in the presence of *Propionibacterium acnes*. *P. acnes* liberated a lipase that was detected on Tween 80 agar and also on phospholipase C-precipitated egg yolk agar. Such a lipase might have contributed in the process of an intensified cellular lysis. Similar reactions were attempted with *Lactobacillus acidophilus*, known to possess a nondiffusible lipase, and failed to produce any such reactions. The synergistic reactions, between *P. acnes* and *C. perfringens*, were compared with the classical CAMP reaction in an attempt to find a correlation with the established membrane composition of the erythrocytes involved. Synergistic reactions observed do seem to reflect the membrane composition. Such findings, besides being contributory to an understanding of the role of these organisms in the process of pathogenesis, are of importance in the elucidation of molecular organization of biomembranes. Detailed studies, involving a large number of representative anaerobic bacteria, may also help provide an avenue in anaerobic species identification.

During routine anaerobic subcultivation of a strain of *Clostridium perfringens* and of a *Propionibacterium acnes* strain on singular sheep blood agar (SBA) half-plates, a vertical column of intensified lysis of erythrocytes was observed in the adjacent growth area of the two subcultures (see Fig. 1). *P. acnes* alone, on prolonged incubation, has a weak lytic effect on these cells (4), indicating the lack of abundance or the absence of an enzyme that would be active on intact, phospholipid-rich erythrocytes. On the other hand, the presence of a propionibacterial glycerol ester hydrolase (lipase) could be envisaged that would, theoretically, act on phospholipase C- or sphingomyelinase-treated erythrocytes, thus bringing about their thorough lysis. A ready source of sphingomyelinase and phospholipase C are found in the organisms *Staphylococcus aureus* (beta lysin) and *C. perfringens* (alpha toxin), respectively. Circumstantial evidence is presented in this paper in favor of a lipase-induced complete lysis (propionibacterial) of erythrocytes partially processed either by *S. aureus* or *C. perfringens*.

MATERIALS AND METHODS

Bacterial strains. *P. acnes* ATCC 6919 was gathered from the American Type Culture Collection, Rockville, Md. *S. aureus* ATCC 25923 originated from the Difco Laboratories, Detroit, Mich. Foothills Provincial Laboratory of Calgary, Alberta, Canada, provided the strain of *Lactobacillus acidophilus* NCTC 1899, whereas the strain of *C. perfringens* CBH-CPER-1 and the strain of *Streptococcus agalactiae* CBH-SAGA-1 are clinical isolates of Colonel Belcher Hospital, Calgary, Alberta, Canada.

Media and gaseous phase. Sheep or human (O, Rh positive) blood agar (10%) was prepared by adding the required amount of respective types of whole blood to BBL blood agar base medium (BBL, Cockeysville, Md.). Five percent egg yolk agar or 0.5% Tween 80 agar was prepared by similarly supplementing the commercial blood agar base with either egg yolk or Tween 80 syrup (Fisher Scientific Co., Pittsburgh, Pa.). The foregoing media were dispensed in 20-ml amounts into standard Fisher plastic petri dishes (100 by 15 mm) or into Fisher Integrid petri dishes. Brain heart infusion (BHI) was prepared according to the manufacturer's instructions (BBL).

Anaerobic atmosphere was maintained by utilizing the BBL GasPak (no. 70304) system.

Procedure. To determine the pH change brought about by the 48-h growth of an organism, 20 ml of BHI, in 13- by 125-mm tubes, was inoculated with a loopful (3 mm) of growth (48-h-old sheep blood agar culture) of the organisms under study. For hemolytic and lipolytic studies, similar 48-h-old growths of the relevant organisms were inoculated adjacent to each other on respective indicator media (Fig. 1 and 2). To quantitate the synergistic hemolytic activity, *P. acnes*, *S. agalactiae*, or *L. acidophilus* was applied with the heads of applicator sticks (diameter, 2 mm) to a blood agar surface directly opposite (10 mm) similarly applied *C. perfringens* or *S. aureus*. Three such pairs could be accommodated on a single standard petri dish. Such plates were incubated for 48 h, anaerobically, at 37°C. Lunar-shaped, clear areas that devel-

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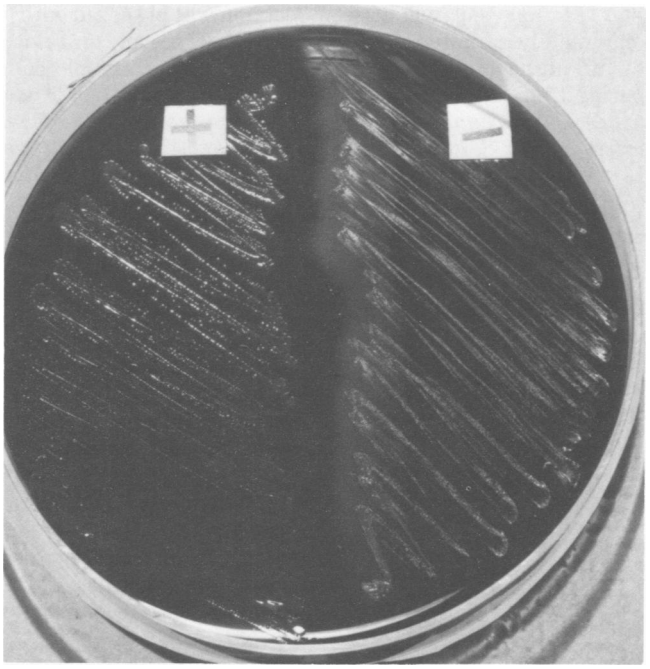

FIG. 1. *Augmentation of lysis of sheep erythrocytes by P. acnes. (+), 48-h growth of C. perfringens CBH-CPER-1; (−), growth of P. acnes during the same period.*

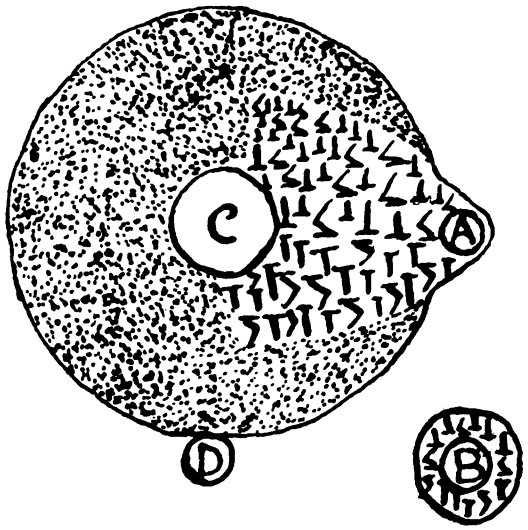

FIG. 2. *Lipolytic activity of P. acnes, in the proximity of C. perfringens, on egg yolk medium. (A) P. acnes ATCC 6919; (B) S. aureus ATCC 25923; (C) C. perfringens CBH-CPER-1; (D) L. acidophilus NCTC 1899. Stippled area surrounding the growth of C. perfringens represents the precipitation zone within the medium caused by the activity of clostridial lecithinase. Parabolic area on the surface of the medium represents the liberated free fatty acids, caused by the activity of propionibacterial lipase.*

oped in the middle of the aforementioned paired inoculated sites were carefully cut and placed on graph paper. The outline of such agar pieces were delineated with a sharp pencil, and their areas were measured. The relative synergistic hemolytic activity of *P. acnes* and *S. agalactiae* on sheep or human blood in the presence of *C. perfringens* or *S. aureus* is expressed as the ratio of the area lysed by *P. acnes* to that of *S. agalactiae*. Triplicates were maintained for each treatment, and the results were averaged for the purpose of presentation.

In certain instances, live culture of *C. perfringens* was substituted by pure clostridial phospholipase C (5×10^{-4} U in 0.05 M phosphate buffer of pH 7.4; Sigma type I, Sigma Chemical Co., St. Louis, Mo.). For the purpose of the specific inhibition of the activity of the phospholipase C, Wellcome *C. perfringens* type A anti-alpha antiserum was used (Wellcome Reagents Ltd., Beckenham, Kent, England).

RESULTS

Centrifuged supernatants, originating from the 48-h growth of *P. acnes, C. perfringens, L. acidophilus, S. aureus,* and *S. agalactiae* in BHI, gave pH readings of 7.2, 6.7, 5.2, 6.2, and 6.2, respectively.

An example of synergistic lysis of sheep erythrocytes, brought about by *P. acnes* in the presence of *C. perfringens,* is depicted in Fig. 1. Similar results could be obtained with purified clostridial phospholipase C at a concentration of

5×10^{-4} U. Synergistic reactions, such as with *C. perfringens,* were completely inhibited in the presence of clostridial anti-alpha antiserum.

The results, originating from the studies on lytic activity of *P. acnes* and *S. agalactiae* in the presence of *C. perfringens* or *S. aureus,* are presented in Table 1. It would appear from the results that both *P. acnes* and *S. agalactiae* were effective to the same degree in lysing the *C. perfringens*-processed sheep cells, thus yielding a ratio of 1. In contrast, with *C. perfringens*-processed human cells, a ratio of 20 was obtained due to the higher synergistic activity of *P. acnes* and the absence of such activity with *S. agalactiae.* When sheep cells were processed by *S. aureus, P. acnes* was less effective in lysing the sheep erythrocytes than *S. agalactiae.* Human erythrocytes (HRBC), similarly processed by *S. aureus,* were not affected by either *P. acnes* or *S. agalactiae.*

L. acidophilus, unlike *P. acnes* or *S. agalactiae,* did not induce any lysis of any of the erythrocyte types studied in the presence of either *C. perfringens* or *S. aureus.* Neither was there any induction of lysis when *C. perfringens* and *S. aureus* were grown adjacent to each other.

S. agalactiae, L. acidophilus, and *P. acnes,* while growing alone on egg yolk medium for a period of 48 h, did not produce any reaction on this medium. However, when *P. acnes* was growing near the *C. perfringens*- or *S. aureus*-precipitated egg yolk medium, a pearly area, in the shape of a parabola, was noted on the surface of the medium (Fig. 2). The vertex of such a parabola was pointing towards the growth of *P. acnes.* No such reaction was observed with either *L. acidophilus* or *S. agalactiae. C. perfringens* alone during this period of incubation (48 h) produced only a precipitation reaction in the egg yolk medium, whereas *S. aureus* gave rise to precipitation reaction and the formation of a narrow pearly collar.

On Tween 80 agar, and on immediate removal of the plate from the anaerobic atmosphere, no reaction was observed with any of the organisms studied. However, on reincubation of the plate at 37°C, under aerobic conditions, a precipitation reaction and the formation of a pearly layer were observed with *P. acnes* at 4 h and with *S. aureus* at 24 h. No such reaction was apparent with *S. agalactiae, L. acidophilus,* or *C. perfringens.*

TABLE 1. *Synergistic lysis of erythrocytes by P. acnes and S. agalactiae in the presence of C. perfringens or S. aureus*

Type of blood	Ratio of the areas lysed (*P. acnes-S. agalactiae*) in the presence of[a]:	
	C. perfringens CBH-CPER-1	*S. aureus* ATCC 25923
Sheep	1 (13.5/12.3)	0.7 (6.5/9.0)
Human	20 (20/-)	- (-/-)

[a] -, Absence of synergistic lysis; figures within parentheses represent the areas of synergistic lysis in terms of the smallest number of squares (0.1 by 0.1 inch) covered on a graph paper.

DISCUSSION

C. perfringens alpha toxin (phospholipase C) and *S. aureus* beta hemolysin (sphingomelinase) are both "hot-cold" hemolysins; i.e., relevant erythrocytes pretreated with these enzymes become sensitive to physical shock, such as sudden change in temperature, pH, or osmolarity (10). These observations imply that the removal of phosphorylcholine moieties alone from the relevant phospholipids does not destroy the integrity of the erythrocyte membrane insofar as the release of hemoglobin is concerned. Of the foregoing factors mentioned, the pH factor needs consideration because of the potentially significant change in this factor that may occur during the organism's growth. It would appear, from the results on pH changes brought about by the organisms in BHI over a period of 48 h, that this factor did not play a significant role in inducing synergistic lysis of the erythrocytes. This is reflected in the less dramatic changes in pH that are observed at the end of the incubation period. This notion is also confirmed from the observations with *L. acidophilus.* The organism causes a maximum change in pH during its growth in BHI, and yet fails to induce any synergistic lysis of the erythrocytes studied.

Besides the aforementioned physical agents, which augment the lysis of the phospholipase-processed erythrocytes, presence of another enzyme, complementary to the activity of the phospholipases under proper substrate distribution, can also lead to an enhanced cellular lysis. In the present study, such an enzymatically synergistic mechanism seems to be operating in the intensified lysis of erythrocytes during the presence of *P. acnes* or *S. agalactiae.* Clostridial phospholipase C or staphylococcal sphingomyelinase, presumably, removed the phosphorylcholine portions of its respective substrates present in the erythrocytes (3), leaving behind the diglyceride and the ceramide residues to be acted upon by a glycerol ester hydrolase (lipase). With *P. acnes,* liberation of such a lipase is evident from the presence of free fatty acids over the *C. perfringens*- or *S. aureus*-precipitated zone of the egg yolk medium. The pearly appearance over the reaction site is due to interference of the light rays by the hydrophobic portions of the liberated fatty acids that rose to the air-water interface of the medium. The presence of

such a propionibacterial esterase (presumably, a variant function of the same lipase [6]) is further confirmed by the appearance of a pearly layer on Tween 80 agar following the growth of *P. acnes*. This layer, apparently, is entirely composed of oleic acid molecules, originating from the hydrolysis of the substrate, Tween 80. A lipase of *P. acnes* that is active on trigycerides has been isolated and partially purified (9). Failure to observe any lipase activity, and therefore an absence of synergistic hemolytic activity, with *L. acidophilus* may have been due to the intracellular nature of this enzyme in this organism (6). Although no lipase activity could be demonstrated in the present study with *S. agalactiae,* the lytic factor of this organism could be a lipase with a superior specificity for ceramide. Such a clue is, perhaps, suggested in the finding that synergistic activity of *S. agalactiae* is superior with *S. aureus*-processed sheep cells. As much as 50% of the total phospholipids of the erythrocyte membrane of this species is comprised of sphingomyelin, with hardly any lecithin (1). It is also known that staphylococcal sphingomyelinase hydrolyzes sphingomyelin most rapidly and does not hydrolyze phosphatidylcholine and phosphatidylethanolamine (10). Such being the case, then it appears that with *S. aureus*-processed sheep cells there would mainly be ceramide residues in the membrane that are to be acted upon by a relevant enzyme so that a synergistic lysis of the cells may occur. Both *P. acnes* and *S. agalactiae* can be speculated to provide such an enzyme in the form of lipases. A lipase that is active on triglycerides has been observed with a strain of *Streptococcus diacetilactis* (6).

Superior synergistic activity with *P. acnes* and *C. perfringens* against HRBC and the absence of any such reaction with staphylococci-processed HRBC could be explained on the basis of two assumptions: first, that the propionibacterial lipase has broader specificity for substrates than streptococcal lipase, and second, that for any lipase (glycerol ester hydrolase) to be effective in the process of hemolysis greater than 25% of the total phospholipids of any erythrocyte membrane are to be preprocessed by a phospholipase. For example, HRBC contains lecithin, sphingomyelin, and phosphotidylethanolamine in equivalent proportions (8) and is susceptible to the action of clostridial phospholipase C in a similar sequential order (2). Therefore, one notices greater synergistic activity against HRBC involving *P. acnes* and *C. perfringens*. With staphylococcal sphingomyelinase, however, only sphingomyelin (constituting 25% of the total phospholipids of HRBC) will be processed, and, therefore, no synergistic activity would be observed with any of the lipases. Perhaps removal of such a small proportion of phosphorylcholine moieties of the phospholipids does not allow the entry of the lipases, or perhaps the neutral lipids so released are insufficient to saturate the relevant enzymes.

Literature indicates that certain members of the skin flora, such as *P. acnes* and *Staphylococcus albus,* can no longer be dismissed as harmless commensals. Free fatty acids, liberated in the pilosebaceous follicle and the sebaceous duct due to the activity of lipases originating from these organisms, act as irritating agents, leading to the inflammatory condition of acne (7). Besides the pathogenic role of *P. acnes* lipase, it appears from this study that an additional pathogenic role can be attributed to this microbial enzyme, i.e., augmented mammalian cellular lysis that has been preprocessed by a mutually complementary bacterial phospholipase. Such studies are of value not only from the clinical point of view, as has been pointed out by Bernheimer (1), but also are of importance in elucidating the molecular organization of biomembranes.

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